Development of fluorescent cholesterol derivatives for the exogenous introduction of proteins to the plasma membrane

PhD thesis

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1. Intorduction

In living systems the membrane-associated proteins play essential roles in the maintenance of the normal life functions. Membrane proteins have various functions as there are membrane receptors, transport proteins, enzymes and cell adhesion molecules among them. Membrane proteins are associated with lipid bilayers in different ways, and they can be classified as integral, peripheral and lipid-anchored proteins. In this latter case the proteins anchored to cell membrane through saturated or unsaturated fatty acid chains including *S*-prenylation and *S*-palmitoylation of Cys residues, *N*-myristoylation of *N*-terminal Gly residues and *C*-terminal amidation with glycosylphosphatidylinositol (GPI) glycolipids (Figure 1). The amidation of a protein *C*-terminus with a GPI glycolipid results in proteins that are tethered to the extracellular leaflet of the cell membrane. The GPI anchor molecule is a glycosylated phosphatidylinositol with stearyl chains, the glycan core consists of a glucosamine and three mannoses, and also contains a terminal ethanolamine phosphate. The GPI-APs are able to temporarily associate with sphingolipid- and cholesterol-rich membrane microdomains, i.e. lipid rafts. Beyond the normal physiological functions, GPI-APs are associated with a range of diseases including paroxysomal nocturnal hemoglobinuria, carcinogenesis and sleeping sickness and the pathobiology of trypanosomal parasites and prion diseases. The function of the cellular prion protein (PrP^{C}) is not well understood, however its β-structure-rich isoform (PrP^{Sc}) that is formed by conformational changes is responsible for the fatal transmissible spongiform encephalopathies. Several studies provide substantial evidences that the GPI anchor of the PrP plays an important role in the formation of the pathological prion conformer. The GPI pool of the prions is heterogeneous; the species differ in the oligosaccharide structure and in the lipidation level. Therefore a simplified mimetic molecule serie was designed to achieve the function of the GPI anchor, which is presumably able to target the PrP^{C} into lipid raft membrane structures.

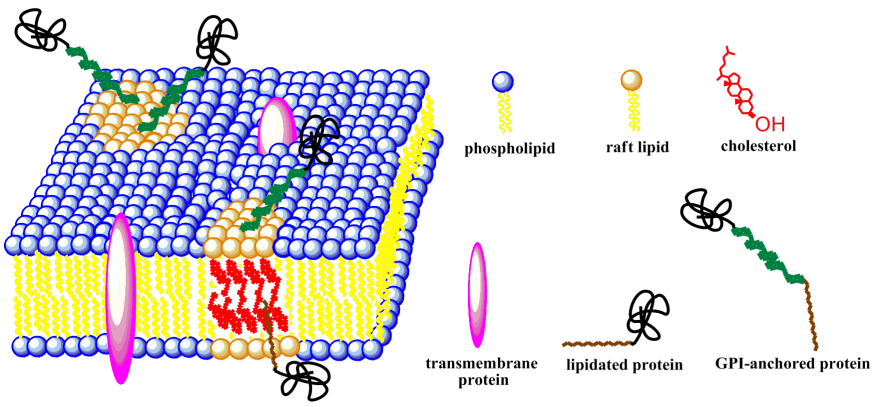

Figure 1. Representative section of the plasma membrane.

2. Aims of the study

1. Synthesis of fluorescent cholestrol derivatives for mimicking GPI anchors.
2. Optimization of the protein conjugation of water soluble cholesterol anchor.
3. Investigation of the cell membrane delivery of fluorescent cholesterol anchors and that of their protein conjugates.
4. Quantitation of the membrane delivered cholesterol anchor by using a tritium labeled analogue.
5. Anchoring the full length prion protein to cell membrane with a fluorescent cholesterol anchor.
6. Application of the cholesterol anchor in cell-surface protein conjugations.

3. Experimental methods

The purity of all reagents and solvents were analytical or the highest commercially available grade. Analytical thin layer chromatography (TLC) was performed on 5×10 cm glass plates precoated with silica gel 60 F_{254}, spots were visualized with UV light, ninhydrin or phosphomolybdic acid. Flash chromatography was carried out on silica gel 60 using the indicated solvents. Analytical HPLC separations and semipreparative HPLC purifications were performed with a Merck-Hitachi LaChrom system. Specific optical rotation was determined with an Optical Activity AA-5 automatic polarimeter at 589.4 nm.

The structure of the compounds were determined by measuring 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC) NMR spectra which were recorded on a Bruker Spectra DRX 500 MHz spectrometer. Proton and carbon assignment of the cholesterol moiety was based on literature data. Molecular weight of the compounds was determined by ESI-MS analysis on a Finnigan TSQ 7000 or on a Bruker reflex III MALDI-TOF spectrometer. FT-IR spectra were recorded with a Bio-Rad Digilab Division FTS-65A/896 FT-IR spectrometer in the 4000–400 cm^{-1} interval at 4 cm^{-1} optical resolution using a diamond ATR probe or in KBr pellets.

Fluorescence excitation and emission spectra were recorded on an Agilent Cary Eclipse fluorescence spectrophotometer. ECD spectra were recorded on a Jasco J815 spectropolarimeter, equipped with a Peltier temperature controller, at 25°C and 100 nm/s scan speed using a 1 mm path-length quartz cell. Protein concentration of the samples was in the 2.5 – 6.5 μM range as it was measured by the Bradford method. Spectra presented in the thesis are accumulations of 10 scans and the corresponding solvent spectra recorded under the

same conditions were subtracted. The contribution of each secondary structural element of the proteins was determined by deconvolution of the spectra using the CDSSTR method.

For *in vitro* biological studies, SH-SY5Y (ATCC: CRL-2266) human neuroblastoma cells were cultured in DMEM medium containing 10% FCS, L-glutamine (2 mM), gentamycin (160 μg/mL), 1 mM pyruvate and non-essential amino acids. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells treated with the fluorescent anchors or protein conjugates were visualized with an Olympus IX81 confocal laser scanning microscope and FluoView 500 software. For fluorescent membrane staining 20000 cells per well were plated on 8-well Lab-Tek II Chambered coverglass. After 48 h incubation at 37°C, cells were treated with the anchors or the protein-anchor conjugates, dissolved in serum-free medium for 30 min. After treatment and incubation, cells were washed with serum-free medium and the nucleus of the cells was stained for 5 min using the nuclear dye DRAQ5.

4. Summary of the results

4.1. Molecular design, synthesis and optimization of fluorescent cholesterol anchors

The main goal of this work was to develop simplified GPI mimetic lipids and to establish a method for their protein conjugation and for the exogenous introduction of the semisynthetic lipoproteins to the plasma membrane of live cells. The resulting lipidated proteins are useful tools to investigate membrane anchored proteins, which have important role in the maintenance of the normal life functions as well as in some diseases.

In order to achieve our goals, fluorescent cholesterol derivatives were designed and synthesized. The application of cholesterol as the membrane anchoring hydrophobic moiety of the GPI substitutes, anticipates the direction of the protein conjugate to the cholesterol-rich lipid rafts, which is the accumulation platform of GPI-APs. In a convergent synthetic strategy propargyl glycine (Pra) was used as an orthogonal trifunctional linker to build up the headgroup of the cholesterol anchors, and separately, amphiphilic cholesterol derivatives were prepared from cholesteryl hemisuccinate and amino-oligo(ethylene glycol) azides. In order to obtain relevant GPI substitutes, the length of the polyether linker was chosen to be similar to that of the glycan core of the GPIs, because this hydrophilic moiety is responsible to expose the attached protein to the extracellular space (Figure 2). A fluorescent reporter and a Michael acceptor were introduced via the transformation of the amino and the carboxyl groups of Pra. The Michael acceptor was exploited for the conjugation with C-terminally Cys-extended

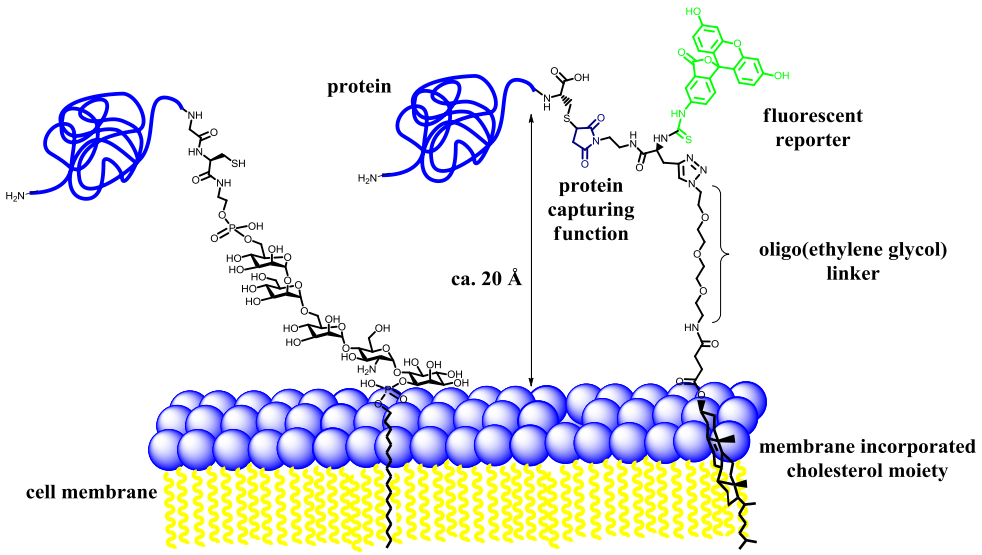

Figure 2. Comparison of the GPI anchor with a designed semisynthetic analogue.

proteins, while direct fluorescence microscopic studies of the membrane-associated cholesteryl lipoprotein conjugates required the small molecule fluorophores. Finally, the fluorescent anchors were assembled in an azide-alkyne cycloaddition.

The physicochemical properties of the cholesterol anchors were optimized by the introduction of three different fluorescent reporters (fluorescein, BODIPY and Cy5) with different polarities and spectral features (Figure 3). The usefulness of the anchors was estimated by fluorescence microscopic investigation of their capability to stain the plasma membrane of SH-SY5Y cells. The solubility of the cholesterol anchors in aqueous buffers was crucial both for the effective protein conjugation and for the exogenous introduction of the resulting cholesteryl lipoproteins into live cell membranes. It was found that the hydrophilic character of the fluorophore deeply affected the water solubility of the anchors,

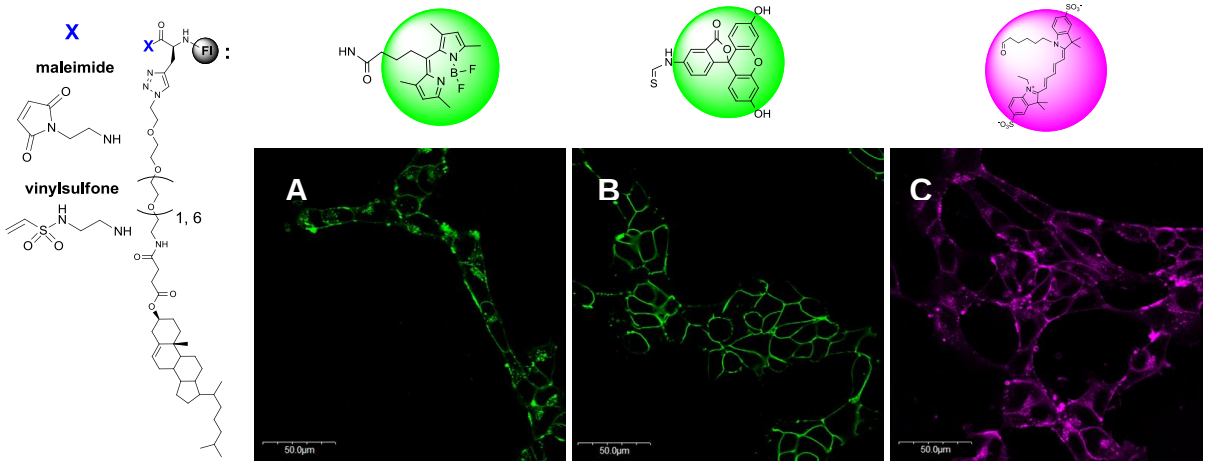

Figure 3. Association of 1 µM A) BODIPY-labelled anchor in β-cyclodextrin inclusion complex and 1 µM B) fluoresceine-labelled anchor and 10 nM C) Cy5-labelled anchor with SH-SY5Y cell membranes.

while increasing the length of the polar oligoether spacer has minor effect. The BODIPY-labelled cholesterol anchors exhibited the least solubility in polar solvents however homogeneous membrane staining was achieved when it was applied in a β-cyclodextrin inclusion complex. In the case of the fluorescein-labelled anchors homogeneous cell membrane staining was observed without the application of β-cyclodextrin when the phenol of the fluorescein moiety was converted into diisopropylammonium phenolate salt. However, the pH dependent fluorescence and the low photostability of fluorescein inspired us to consider the incorporation of sulfonated Cy5 which is highly polar and exceptionally bright. It was found that the Cy5-labelled cholesterol anchor resulted in an intensive homogeneous membrane staining at lower concentration than the fluorescein-labelled derivative.

Two different Michael acceptors, a maleimide and a vinylsulfone were investigated for the protein conjugation. Their reactivity and selectivity differs, which is important when proteins requiring different buffer conditions for solubilization are to be lipidated. The Michael additions were optimized by using a Cys-containing model peptide, and it was found that the maleimide was more reactive at lower pH, while the vinylsulfone was more selective at higher pH toward the Cys thiol Michael donor.

Before the application of the anchors for protein cell membrane delivery, the hydrolytic stability of the headgroup was also investigated. The crucial cholesteryl ester was found to be stable against hydrolysis at physiological pH. Furthermore, the membrane fluorescence remained constant during long-term incubation of the cell membrane incorporated fluorescent anchors suggesting that the headgroup of the anchors were not subjected to enzymatic hydrolysis.

4.2 Semisynthesis of cholesteryl mCherry and its cell membrane association

The protein conjugation of the fluorescent anchors was investigated via Michael addition to the C-terminally Cys extended, recombinant mCherry-Cys protein. The mCherry is a red fluorescent protein and its conjugation with a green fluorophore containing cholesterol anchor results in a dual fluorescent lipoprotein conjugate. When it is delivered into the cell membrane, the appearance of both red and green fluorescence on the plasma membrane indicates the successful membrane anchoring of the protein without significant change in the protein structure. The mCherry-Cys – cholesterol anchor addition resulted in a mixed micellar associate of the protein–anchor conjugate, because the amphiphilic cholesterol anchor dissolves in aqueous buffers in micellar form. A two-step purification method was then optimized to obtain anchor excess-free cholesteryl mCherry that includes the application of β-

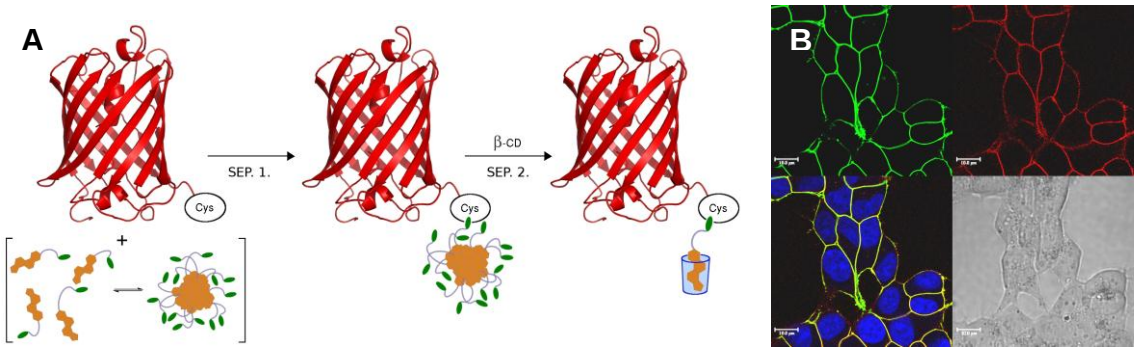

Figure 4. (A) Preparation of the lipid excess-free cholesteryl mCherry. The schematic green heads represent the green fluorophore of the cholesterol anchor, β-cyclodextrin is symbolized with a blue truncated cone, SEP.1. and SEP.2. indicate gel chromatographic separations and B) Confocal laser scanning and differential interference contrast (right bottom) microscopy of SH-SY5Y cells after 30 min incubation with *β*-cyclodextrin inclusion complex of 1 μM cholesteryl mCherry at 37°C. On the images fluorescein is green, mCherry is red and nuclei are blue. On the overlayed image (A and B, left bottom) yellow represents the colocalization of the fluorescein and mCherry signals.

cyclodextrin to disrupt the micellar associates. In the resulting β-cyclodextrin inclusion complex of cholesteryl mCherry, the lipid part of the conjugate remained soluble in cell culture media and the protein was not denatured. The purity of the dual fluorescent cholesteryl mCherry was evidenced by SDS-PAGE, and the preserved protein structure was revealed by CD spectroscopy. The confocal microscopy images, especially the colocalization of the green and red fluorophore confirmed that the mCherry protein was anchored to the plasma membrane of live cells via the green fluorescent cholesterol anchor (Figure 4).

4.3 Conjugation of the full length prion protein with cholesterol anchors

The most investigated GPI anchored protein, prion protein (PrP) was chosen to modify with the synthetic cholesterol anchors and to investigate if cholesteryl PrP derivatives are appropriate for cell membrane delivery. The importance of such a model system is that the biological function of the PrP is not known, the details of the conformational changes of this membrane-anchored protein resulting in prion diseases are unrevealed, and the role of the PrP GPI anchor in these processes is also unclear. Therefore, the recombinant full length mouse PrP was conjugated to cholesterol anchors and after an optimized semisynthesis and purification, the introduction of the cholesteryl-PrP was achieved into the plasma membrane of live neuroblastoma cells (Figure 5). The purity of the fluorescent cholesteryl PrP was evidenced by SDS-PAGE, and the protein structure was revealed by CD spectroscopy.

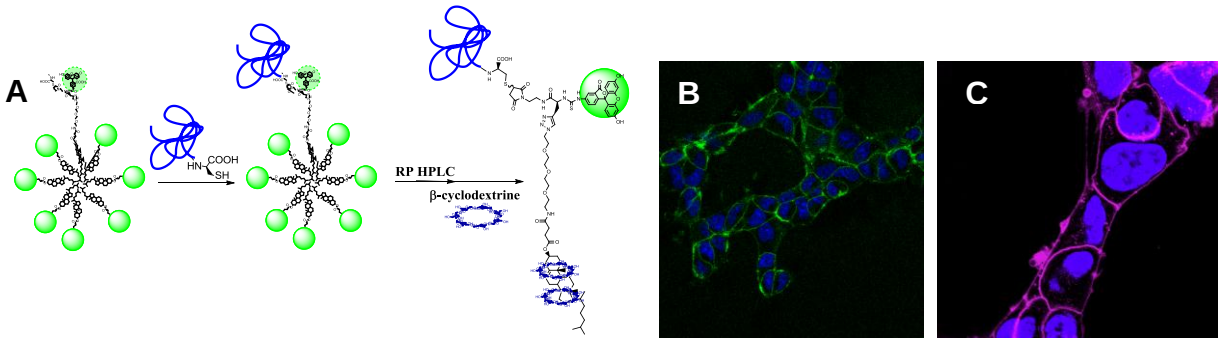

Figure 5. A) Preparation of mPrP–fluorescent-anchor. B) Confocal laser scanning microscopy of SH-SY5Y cells after 30 min incubation with 3 μM S231C mPrP(23-231)–fluoresceine-anchor, C) 10 nM S231C mPrP(23-231)–Cy5-anchor.

4.4 Quantitation of the cell membrane delivery of cholesterol anchors

In order to quantitate the cell membrane delivered cholesteryl lipoproteins, a tritium labelled cholesterol anchor was prepared. Similarly to the fluorescent labelling, the radioactive reporter was introduced into the headgroup of the anchor. When SH-SY5Y cells were incubated with the [^{3}H]cholesterol anchor, a concentration and time dependent membrane incorporation was observed (Figure 6).

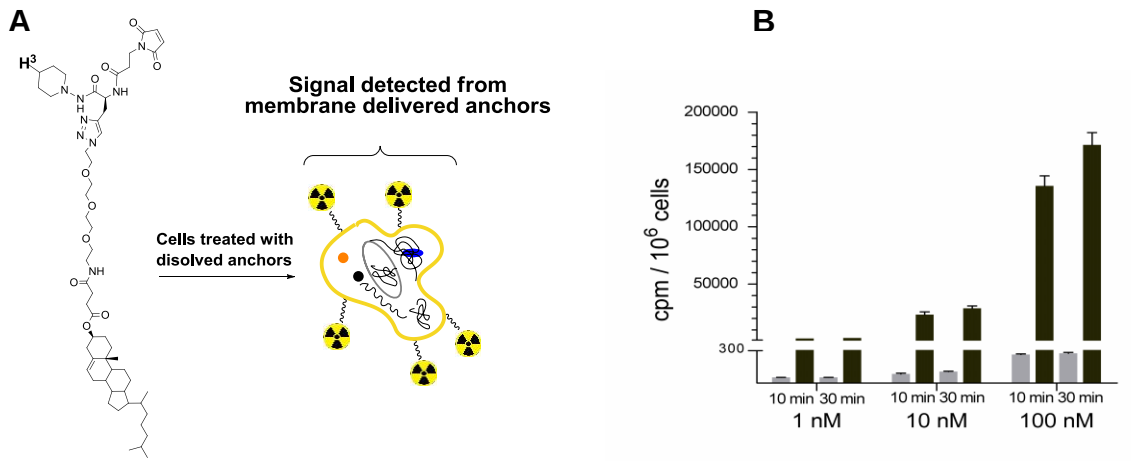

Figure 6. A) Tritiated anchors dissolved in cell culture media are added to cells. After incubation, the cells were washed with fresh media and the cell membrane incorporated anchors remained in bilayer. After that the radiation is detectable from membrane delivered anchors. (B) Incorporation of the [^{3}H]cholesterol anchor to the membrane of SH-SY5Y cells. Cells were incubated with different concentration of the [^{3}H]aminopiperidine (grey bars) or the [^{3}H]cholesterol anchor (black bars) in the presence of 10 μM β-cyclodextrin followed by liquid scintillation counting of the washed and trypsinized cells. The error bars represent SEM values of three parallel measurements. In 100 nM concentration with 30 min incubation the results suggested that 2×10^6 [^{3}H]cholesterol anchor molecules were incorporated into SH-SY5Y cells.

4.5 Protein anchoring to the plasma membrane with cell surface click reaction

In order to eliminate the cholesteryl lipoprotein purification steps from our strategy and to minimize the risk of the protein denaturation, an alternative convergent semisynthetic method was investigated. This method is a cell surface application of the CuAAC reaction including the conjugation of a maleimido fluorescent Pra linker to the protein C-terminus followed by a cycloaddition through the alkyne to an azido cholesterol pre-incorporated into the cell membrane. In this way the target fluorescent cholesteryl lipoprotein was formed *in situ* on the surface of live cells. Similarly to the β-cyclodextrin mediated membrane delivery, surplus detergents or unreacted fluorescent lipids were not introduced into the plasma membrane, and thus, the fluorescent signal is unambiguously associated with the cell surface-prepared cholesteryl lipoprotein. By using this method the structure sensitive red fluorescent protein, mCherry was successfully anchored to the plasma membrane of live cells (Figure 7). The application of His and aminoguanidine as accelerator ligands protected live cells from the cytotoxic effects of the CuAAC catalyst components, and under these conditions the mCherry protein was not significantly denatured.

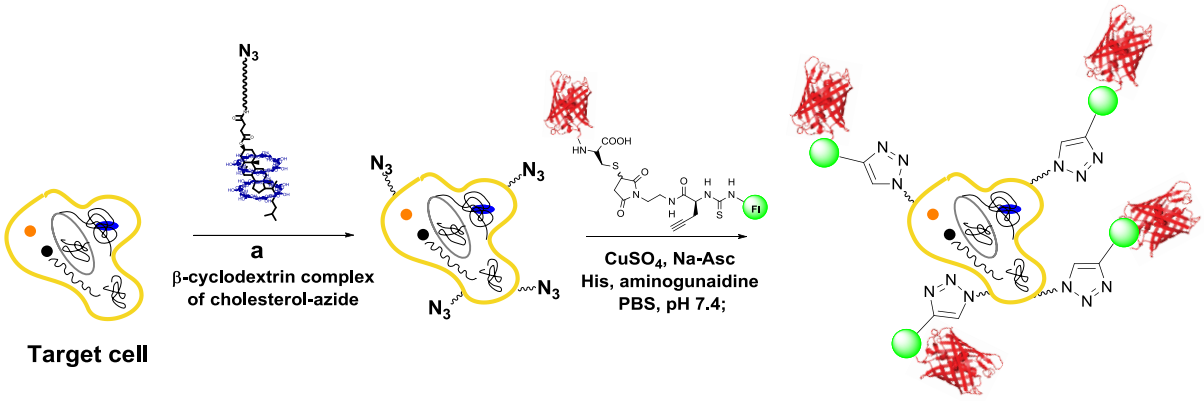

Figure 7. Schematic representation of the cell surface CuAAC. SH-SY5Y cells were azido-cholesterol loaded with a (a) 30 μM cholesterol azide–β-cyclodextrin inclusion complex-treatment in serum free medium, RT, 30 min, followed by incubation with 60 μM mCherry-fluorescent alkyne conjugate in the presence of 50 μM $CuSO_4$, 500 μM NaAsc, 100 μM His, 500 μM aminogunaidine, PBS (pH7.4), 30 min.

5. Conclusion and final remarks

In this work some fluorescent GPI anchor mimetics were synthesized and optimized for protein conjugation. The protein-anchor conjugates were purified and then the lipoproteins were anchored to live cell membrane without denaturing the native protein.

For further studies of GPI-APs the quantitation of membrane delivered anchors may have importance therefore a tritium labeled anchor analogue was synthesized and delivered to the cell membrane. The incorporation was concentration and time dependent, and the exact amount of the membrane incorporated anchor was determined from the radioactivity of cell filtrates.

The protein anchoring to cell membrane was studied by cell surface click reaction also, where the consecutive purification steps are acclaimed with the risk of protein denaturation. In this method a fluorescent hydrophilic alkyne containing linker was conjugated to protein and after this conjugation the CuAAC reaction was carried out between the already membrane delivered cholesterol azide and the fluorescent alkyne-protein conjugate.

The main advantage of these methods is that the use of the β-cyclodextrine inclusion complex of the protein conjugate allows the treatment of live cells with fluoresce15nt cholesteryl lipoproteins without the use of membrane perturbing detergents. Furthermore, no surplus fluorescent lipids over the stochiometric cholesterol moiety of the semisynthetic lipoprotein are intoduced into the cell membrane.

Altogether, in this work complete protein cell membrane anchoring strategies were developed for the structural and functional investigation of the cell membrane anchored GPI-APs including PrP.

This thesis based on the following publications:

I. **Schäfer B**., Orbán E., Borics A., Huszár K., Nyeste A., Welker E. and Tömböly C. (2013) Preparation of Semisynthetic Lipoproteins with Fluorescent Cholesterol Anchor and Their Introduction to the Cell Membrane with Minimal Disruption of the Membrane. *Bioconjugate Chemistry 24*, 1684-1697.

II. **Schäfer B**., Orbán E., Kele Z., Tömböly Cs. (2015): Tritium labeling of a cholesterol amphiphile designed for cell membrane anchoring of proteins. *Journal of Labelled Compounds and Radiopharmaceuticals 58,* 7-13.

III. **Schäfer B**., Orbán E., Fiser G. and Tömböly C. (2015) Semisynthesis of membrane-anchored cholesteryl lipoproteins on live cell surface by azide – alkyne click reaction. (submitted)

Posters, published abstracts:

Schäfer, B., Fodor, E., Welker, E., Tömböly, Cs. Synthesis of a glycosylphosphatidylinositol mimetic for prion protein anchoring. Joint Meeting on Medicinal Chemistry, 24-27 June 2009, Budapest, Hungary, pp 217.

Schäfer, B., Fodor, E., Zoltán, K., Welker, E., Tömböly, Cs. Synthesis of a glycosylphosphatidylinositol mimetic molecules. Conference of Hungarian Chemists, 31 June-2 July 2010, Hajdúszoboszló, Hungary. pp 137.

Schäfer, B., Majer, M., Welker, E., Tömböly, Cs. Semisynthesis of membrane associated proteins. Conference of Chemists, 22-25 May 2011, Sopron, Hungary. pp 191.

Tömböly, Cs., Majer, M., **Schäfer, B.**, Welker, E. Semisynthesis of cell membrane anchorable Prion Protein derivatives. 2011. Conference of Chemists, 22-25 May 2011, Sopron, Hungary. pp 72.

Majer, M., **Schäfer, B.**, Welker, E., Tömböly, Cs. Membrane anchoring of the prion protein with synthetic GPI-mimetics. European Conference on Chemistry for Life Sciences, 31 August-3 September 2011, Budapest, Hungary. pp 311.

Schäfer, B., Orbán, E., Vincze, N., Tömböly, Cs. 2012. Preparation of fluorescent cholesterol derivatives as GPI anchor substitutes EMBL Conference, 26-29 September 2012, Heidelberg, Germany. pp 86.

Schäfer, B., Orbán, E., Vincze, Cs., Borics, A., Tömböly, Cs. Protein lipidation with fluorescent cholesterol amphiphils: a useful tool for visualising membrane associated proteins EMBL Conference, 26-29 September 2012, Heidelberg, Germany. pp 268.